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Cox-2 and osteopontin in cocultured platelets and mesangial cells: Role of glucocorticoids

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Cox-2 and osteopontin in cocultured platelets and mesangial cells: Role of glucocorticoids.

Background. Glomerular inflammation is characterized by a consecutive infiltration of immunoreactive cells. To mimic the early phase of glomerular injury, a coculture system of platelets and rat renal mesangial cells was established. As prototypes, the inflammation-related proteins cyclooxygenase-2 (Cox-2) and the chemotactic protein osteopontin (OPN) were investigated.

Methods. The expression of OPN and Cox-2 mRNA and protein was determined by Northern and Western blot analyses.

Results. Coincubation of platelets and mesangial cells led to a rapid, transient induction of Cox-2 mRNA, which peaked at two hours, whereas OPN and monocyte chemoattractant protein-1 (MCP-1) were induced at later time points. The induction of Cox-2 mRNA was concentration dependent and highly reproducible when platelets of different donors were investigated. Partial Cox-2 induction was observed when supernatants of preactivated platelets were incubated with mesangial cells. The inhibition of the signaling pathways of platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) or interference with G_i-protein signaling partially inhibited platelet-induced Cox-2 expression. Down-regulation of protein kinase C (PKC), which is a common signaling module in many pathways leading to Cox-2 induction, almost completely abrogated platelet-induced Cox-2 expression. The time pattern of Cox-2 and OPN expression suggested that Cox-2 might play a role in OPN induction. The up-regulation of OPN was dependent on de novo protein synthesis and was induced by high levels of exogenous prostaglandin E_2 (PGE_2; 10 μmol/L). Endogenous PGE_2, however, proved not to be essential for OPN mRNA expression, because inhibition of Cox activity did not change OPN mRNA levels. Dexamethasone inhibited Cox-2 mRNA induction but increased OPN mRNA and protein expression.

Conclusion. These data indicate that Cox-2 and OPN are independently up-regulated upon interaction of platelets and mesangial cells.

Key words: mesangial cells, platelets, cyclooxygenase, osteopontin, glucocorticoids, injury.

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Renal glomerular inflammation is characterized by infiltration of bone marrow-derived cells, platelets, granulocytes, monocytic cells, and lymphocytes [1]. Regarding the temporal relationship, platelets are among the first cells to infiltrate the kidney transiently, as shown in different animal models, such as Habu snake venom-induced proliferative glomerulonephritis [2], experimental diabetic nephropathy [3], or immune complex nephritis [4, 5]. Glomerular endothelial lesions caused by radiation therapy also led to an influx of platelets into the mesangial space, allowing interaction with mesangial cells [6]. The functional importance of platelet infiltration was shown by treating animals with antiplatelet antibodies. Amelioration of renal function and reduced glomerular cell proliferation was observed in immune complex nephritis [4] and the model of diabetic nephropathy [3]. Depletion of platelets also affected mediator release in acute nephrotoxic serum nephritis in rats [7]. Enhanced synthesis of different types of eicosanoids, prostanoids and leukotrienes, has been shown to be associated with immune-mediated glomerular inflammation, with mesangial cells being the major source of glomerular prostaglandins [8].

Regulation of prostaglandin synthesis in mesangial cells has been studied in detail in vitro. Multiple stimuli, which are potentially released by platelets, are capable of inducing cyclooxygenase-2 (Cox-2), the inducible Cox isoform, among them platelet-derived growth factor (PDGF) [9], lysophosphatidic acid (LPA) [10], thromboxane, or serotonin (5-HT) [11]. Other platelet-derived mediators, however, do not induce Cox-2 expression such as adenosine 5′-triphosphate (ATP), but rather inhibit the induction of the enzyme by other stimuli [11]. The elevation of intracellular cAMP led to a complete inhibition of 5-HT–mediated Cox-2 induction [12], suggesting that prostaglandin E_2 (PGE_2) itself might function as a negative regulator by binding to EP4 receptors, which activate adenylate cyclase [13]. Platelets also release factors such as platelet factor 4 (PF4), which in vitro is able to inhibit mesangial cell proliferation [14].

This suggests an antiproliferative rather than proliferative effect of platelets on glomerular mesangial cells. Caution is thus needed to extrapolate from in vitro experiments using single stimuli to the in vivo situation, in which multiple mediators act in concert and may exert synergistic as well as antagonistic effects. To mimic the in vivo situation more closely, we established a coculture system consisting of platelets and mesangial cells to investigate the regulation of Cox-2, a key enzyme of prostanoid synthesis.

The early infiltration of platelets is followed by infiltration of other cells of the immune system, monocytic cells, or lymphocytes. Activated mesangial cells may enforce this process by secreting chemoattractants such as monocyte chemoattractant protein-1 (MCP-1) [15] or osteopontin (OPN) [16]. OPN is an acidic glycoprotein with cell adhesive and chemotactic properties [17]. In the kidney, OPN has been implicated in macrophage and lymphocyte recruitment to sites of inflammation in experimental models of glomerular and interstitial nephritis [18–20]. In vitro, the up-regulation of OPN has been observed in mesangial cells treated with fetal calf serum (FCS) [16], but also with PDGF or LPA. This suggested that platelets might be able to induce chemotactic factors such as MCP-1 or OPN to regulate the subsequent infiltration of other cells of the immune system. To test this hypothesis, the expression of MCP-1 and OPN was investigated in the coculture system of platelets and mesangial cells.

METHODS

Materials

The tyrphostins AG1296 and AG1478 were obtained from Calbiochem (Bad Soden, Germany). 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was from Serva (Heidelberg, Germany). Stock solutions of AG1296, AG1478, and TPA were prepared in dimethyl sulfoxide (DMSO). Therefore, respective concentrations of DMSO were included in the medium of control incubations. Pertussis toxin (PTX) was from Biomol (Hamburg, Germany). Cell culture reagents were from Biochrom (Berlin, Germany). FCS was from GIBCO (Eggenstein, Germany). Transwell plates (0.4 μm diameter) were obtained from Costar (Cambridge, MA, USA).

Cell culture

Rat mesangial cells were isolated and cultured as described [21]. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mmol/L L-glutamine, 5 μg/mL insulin, 4.5 g/L glucose, 100 U/mL penicillin, and 100 μg/mL streptomycin containing 10% (vol/vol) FCS. Mesangial cells were used between passages 12 and 20.

For the experiments, renal mesangial cells (0.5×10^6 cells/10 mL) were plated in 10 cm diameter Petri dishes in medium with 10% (vol/vol) FCS. At subconfluency (after 3 to 5 days), cells were growth arrested by serum deprivation in DMEM containing 0.5% (vol/vol) FCS for three to four days. Growth-arrested mesangial cells were used in all experiments.

Coincubation of mesangial cells and platelets

Platelets were prepared from venous blood of healthy human donors. For control experiments, platelets were also prepared from the blood of male Sprague-Dawley rats. Platelet-rich plasma was obtained by centrifugation of citrate blood ($190 \times g$, 15 min at room temperature). Platelets were counted, and an appropriate amount of platelet-rich plasma containing the platelets needed to stimulate one Petri dish of serum-deprived mesangial cells (about 10^6 mesangial cells) was pelleted ($2500 \times g$, 4°C, 15 min). The cells were resuspended in 50 μL medium and were immediately transferred to the mesangial cell culture dish.

Northern blot analysis

After stimulation for the times indicated, cells were scraped with a rubber policeman into chilled 50 mL polypropylene tubes and centrifuged ($225 \times g$, 4°C). The pellet was lyzed by adding 300 μL guanidinium isothiocyanate-containing lysis solution. Total RNA was extracted according to the protocol of Chomczynski and Sacchi with minor alterations [22]. RNA yield usually was 30 to 40 μg/10 cm diameter Petri dish. Separation of total RNA (10 μg/lane) was achieved by the use of 1.2% agarose gels containing 1.9% (vol/vol) formaldehyde with 1 × MOPS as gel running buffer. Separated RNA was transferred to nylon membranes by capillary blotting and fixed by baking at 80°C for two hours.

Hybridization was performed with cDNA probes labeled with [^{32}P]-dCTP using the Nonaprimer Labeling Kit II (Oncor Appligene, Heidelberg, Germany) as described [23]. The specific Cox-1 and Cox-2 probes were 2.767 and 1.156 kb EcoRI fragments from the 5′-end of mouse cDNA, respectively [24]. A cDNA probe specific for rat MCP-1 was kindly provided by Dr. T. Yoshimura (NCI, Frederick, MD, USA) [15, 25]. The cDNA probe specific for OPN was obtained as described previously [26]. The GAPDH probe was obtained with a 500 bp reverse-transcribed fragment [11]. Hybridization was performed by standard procedures [10]. DNA/RNA hybrids were detected by autoradiography using Kodak X-OMAT AR film (Eastman Kodak, Rochester, NY, USA).

Quantitative analysis was performed by densitometric scanning of the autoradiographs (Froebel, Wasserburg, Germany). All values were corrected for differences in RNA loading by calculating the ratio of Cox-2 or OPN to GAPDH or 18S rRNA expression.

Western blot analysis

Cellular proteins were isolated using RIPA buffer 850 mmol/L Tris/HCl, pH 7.5, 1% (vol/vol) Triton X100, 0.1% (wt/vol) deoxycholic acid, 0.1% (wt/vol) sodium dodecyl sulfate (SDS), 150 mmol/L NaCl, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium vanadate, 10 mmol/L sodium fluoride, and 3 μg/mL aprotinin. The protein content of cellular lysates was determined with the bicinchoninic acid protein assay (Pierce, Rockford, IL, USA). OPN protein was detected in the cellular supernatants of cells stimulated in serum-free medium. Supernatants (2 mL) were concentrated by Nanosep columns (Pall Filtron, Dreieich, Germany) to about 30 μL. The concentrates were used in total for Western blot analysis.

For Western blot analysis, 30 to 50 μg protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 10% polyacrylamide), transferred onto polyvinylidene difluoride (PVDF) membrane, and probed with a specific antibody directed against Cox-2 (Cayman Chemical, Ann Arbor, MI, USA). The antibody directed against rat OPN was kindly provided by A. Beshensky (Veterans Affairs Medical Center, Milwaukee, WI, USA). The peroxidase-conjugated antirabbit secondary antibody was obtained from Amersham (Braunschweig, Germany). Protein-antibody complexes were visualized by the enhanced chemiluminescence detection system (ECL or ECL plus; Amersham).

Determination of PGE_2

Prostaglandin E_2 was detected in cell culture supernatants by a specific enzyme-linked immunosorbent assay (ELISA) as described previously [27, 28].

DNA synthesis

For the determination of [^{3}H]thymidine uptake, mesangial cells were serum deprived for two days in serum-free DMEM medium. Cells were then stimulated for 24 hours. [^{3}H]thymidine (2 μCi/mL; 2 Ci/mmol; ICN Meckenheim, Germany) was added for the last four hours of the incubation time. Then the cells were washed with PBS, treated with 0.05/0.02% (wt/vol) trypsin/ethylenediaminetetraacetic acid (EDTA), and harvested by a cell harvester. The incorporation of [^{3}H]thymidine was quantitated by liquid scintillation counting.

RESULTS

Induction of mesangial cells proliferation by platelets

Platelets were prepared from the blood of healthy donors and were kept as platelet-rich plasma until immediately before the start of the experiment in order to avoid activation of the platelets and release of soluble mediators. The cells were then spun down and transferred without delay to the serum-deprived mesangial cells. Platelets sedimented and adhered to the mesangial cells. As a functional aspect of platelet–mesangial cell interaction, the effect of platelets on mesangial cell proliferation was determined by measuring the incorporation of radioactive thymidine. Thymidine incorporation was enhanced by increasing numbers of platelets (Fig. 1). The induction of proliferation was concentration dependent and plateaued at a concentration of 20×10^6/mL platelets.

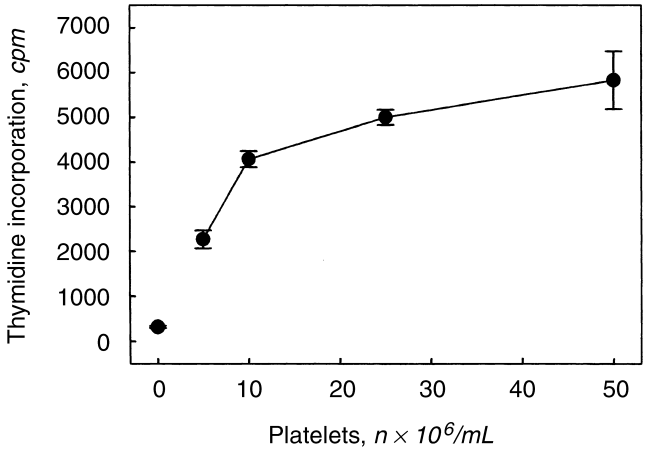


Fig. 1. Platelet-induced proliferation of mesangial cells. Mesangial cells were incubated with different concentrations of platelets for 24 hours. Thymidine incorporation was determined over the last four hours of the incubation time. Data are mean ± SD of triplicates of a typical experiment.

Induction of gene expression by platelet-mesangial cell coincubation

Coincubation of mesangial cells (about 10^6 cells per dish) and platelets (5×10^7 platelets corresponding to 5×10^6/mL) induced the expression of the mRNA of proteins related to inflammation (Fig. 2A). Cox-2 mRNA was up-regulated within the first hour, whereas up-regulation of the mRNAs of the chemotactic proteins OPN and MCP-1 followed later. Gene induction was also observed when rat mesangial cells were incubated with rat platelets (Fig. 2B), excluding an effect caused by surface histocompatibility reactants.

As a control, mesangial cells were incubated with various amounts of platelet-poor plasma that might have remained in the platelet preparation. No effect on mRNA expression was observed with up to 10 μL platelet poor plasma (data not shown). Platelets did not contain detectable amounts of Cox-2 or OPN mRNA: RNA was extracted from 20 and 200 times higher numbers of platelets than those used in the coculture experiments, but no Cox-2 or OPN signal was visible even after longer exposure times (Fig. 2C).

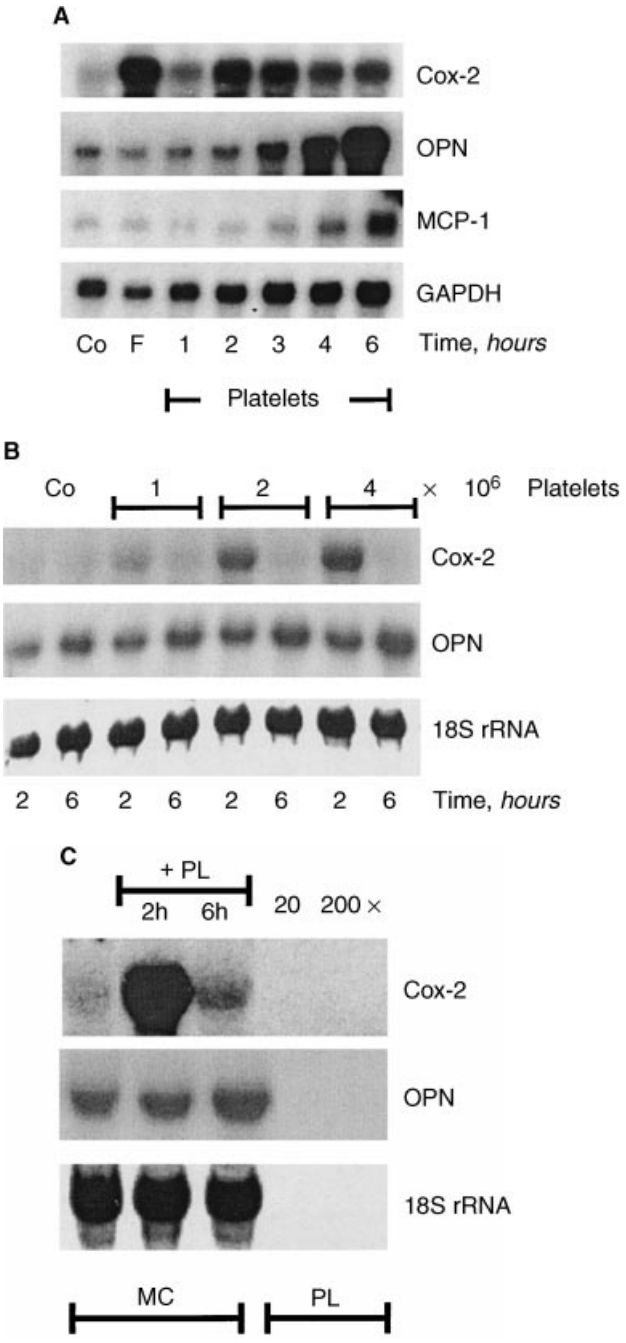


Fig. 2. Time-dependent increase in mRNA expression of inflammation-related proteins. (*A*) Mesangial cells were incubated with FCS (F; 10%) for two hours or with 5×10^6 human platelets/mL for the times indicated. Control cells (Co) were left untreated. mRNA was isolated as described in the **Methods** section. The Northern blot was probed with cDNAs specific for Cox-2, OPN, MCP-1, and GAPDH. (*B*) Rat mesangial cells were incubated with different concentrations of rat platelets for two and six hours. Control cells (Co) were left untreated. The Northern blot was probed with cDNAs specific for Cox-2 and OPN. Ribosomal RNA (18S rRNA) was stained with methylene blue to confirm equal loading. (*C*) RNA was extracted from 20 and 200 times higher numbers of human platelets than those used in the coculture experiment (+ PL), where mesangial cells were cocultured for two and six hours.

Characterization of platelet-induced Cox-2 mRNA expression

We concentrated on the expression of Cox-2 as a very early parameter of platelet–mesangial cell interaction. The induction of Cox-2 was transient with maximal expression of Cox-2 mRNA after two hours (Fig. 3A). The induction was dependent on the number of platelets (Fig. 3B). A linear correlation was obtained between 0.1 and 20×10^6 platelets per mesangial cell dish, corresponding to 0.1 to 2×10^6 platelets per mL medium (Fig. 3C). The incubation of mesangial cells with platelets had no effect on the expression of Cox-1 mRNA. Platelets obtained from different donors were compared in parallel to assess donor variability. The capacity of platelets to induce Cox-2 mRNA was found to be rather similar (Fig. 4). In none of the experiments performed throughout the study were platelets unable to induce Cox-2 mRNA.

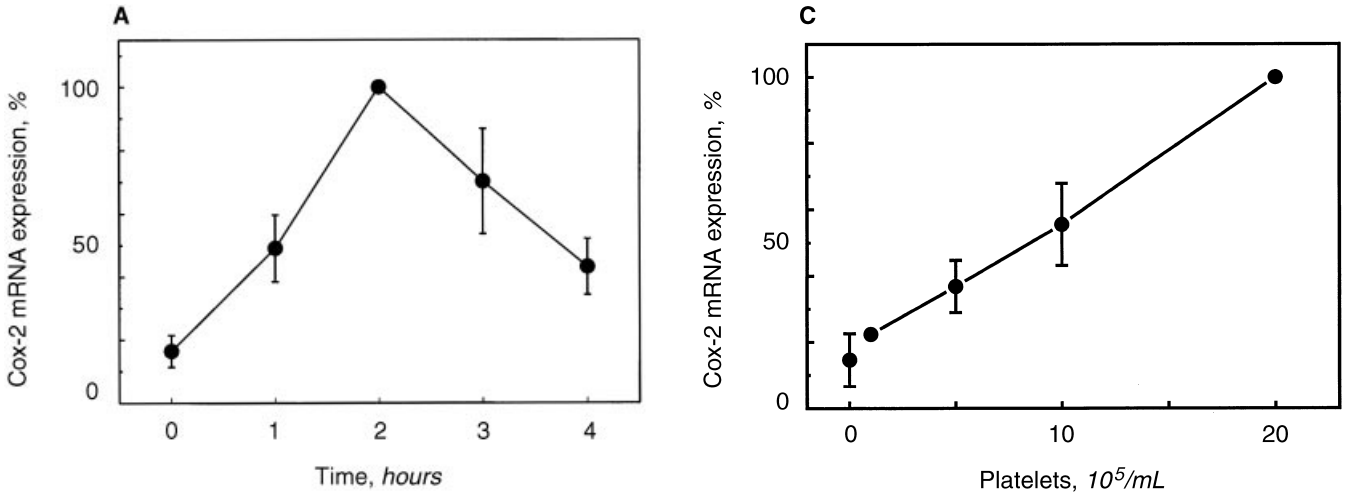
Role of soluble mediators in platelet–mesangial cell interactions

To assess the effect of soluble mediators released by platelets, 2×10^7 platelets were incubated for 30 minutes as platelet-rich plasma or in cell culture medium with 0.5% FCS. To stimulate the platelets, 1 μmol/L adenosine diphosphate (ADP) was added. ADP was chosen as activator because by itself it did not induce Cox-2 mRNA expression in mesangial cells (data not shown). The effect of cell-free platelet supernatants obtained at the end of the incubation period was compared with the effect of platelets coincubated with mesangial cells. As can be seen from Table 1, the induction of Cox-2 mRNA was significantly reduced when only the soluble mediators were transferred. The activation of the platelets in medium was more effective than in plasma.

To further support the importance of direct cell to cell contact, experiments were performed in which platelets were seeded in the upper part of transwell plates, which allowed interaction via soluble mediators but prevented direct contact between platelets and mesangial cells. Compared with the direct interaction, the stimulation of Cox-2 was reduced by 50%, comparable to the effects observed with soluble mediators (data not shown).

Characterization of mediators involved in platelet-induced Cox-2 mRNA expression

To define mediators involved in platelet-induced Cox-2 expression, the signaling pathways of the growth factors PDGF and epidermal growth factor (EGF) were blocked by specific inhibitors tyrphostins AG1296 and AG1478, respectively [29]. Furthermore, mesangial cells were preincubated with PTX to interfere with mediators such as LPA, which induce Cox-2 by binding to receptors that couple to G_i proteins [10]. Inhibition of each pathway led to a partial inhibition of Cox-2 induction (AG1296,



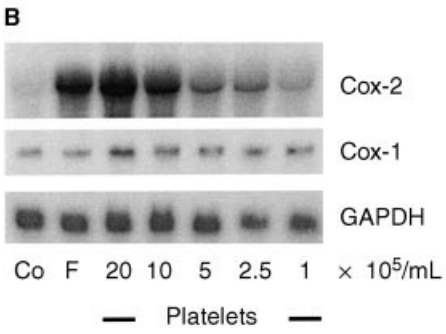


Fig. 3. Characterization of Cox-2 mRNA induction by platelets. (*A*) Mesangial cells were incubated with 2×10^6 platelets/mL for the times indicated. Cox-2 expression was detected by Northern blot analysis. Cox-2 mRNA was quantitated by densitometry and corrected for equal loading using GAPDH expression as a reference. To compare different experiments, stimulation after two hours was set to 100% ($N = 3$, $\pm$ means SD). (*B*) Mesangial cells were incubated with increasing numbers of platelets as indicated for two hours. As control, cells were left untreated (Co) or incubated with 10% FCS (F). A typical Northern blot was probed for Cox-2, Cox-1, and GAPDH. (*C*) Quantitative determination of Cox-2 mRNA expression corrected by GAPDH expression. To compare different experiments, stimulation with 2×10^6 platelets/mL was set to 100% ($N = 3$, mean $\pm$ SD, one experiment with 1×10^5 platelets/mL).

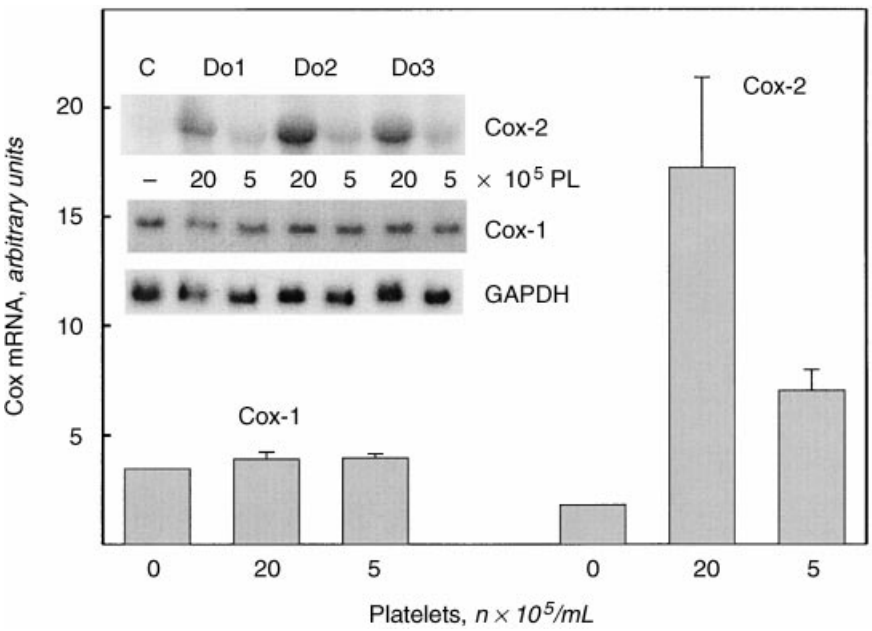


Fig. 4. Donor variability of Cox-2 mRNA expression. Platelets (PL; 20 and 5×10^5 cells/mL) obtained from three different individuals (Do1-3) were incubated with mesangial cells for two hours. The Northern blot was probed with cDNAs specific for Cox-2, Cox-1, and GAPDH (inset). For the quantitative analysis, Cox mRNA expression was corrected by GAPDH expression.

Table 1. Induction of Cox-2 mRNA by soluble platelet-derived mediators

	Cox-2 mRNA expression % ± *SD*
Mesangial cells	5.5 ± 1.3
Mesangial cells + platelets	100
Mesangial cells + platelet-conditioned plasma	20 ± 14
Mesangial cells + platelet-conditioned medium	48 ± 12

Platelets and mesangial cells were either cocultured or mesangial cells were incubated with supernatants of the same number of platelets obtained after a 30-minute incubation period in plasma or cell culture medium in the presence of 1 μmol/L ADP. Cox-2 mRNA expression obtained by coincubation of platelets and mesangial cells was set to 100% ($N = 3$, mean ± SD).

16%; AG1478, 14%; PTX, 34%; means of 2 experiments; Fig. 5A), indicating that not a single mediator but a combination of various mediators is responsible for the observed effect on gene expression. In line with these observations, a stronger inhibitory effect was observed when protein kinase C (PKC) was down-regulated prior to incubation with platelets. PKC has been shown to be an essential part of the intracellular signaling cascade of various stimuli leading to the induction of Cox-2 in mesangial cells [9, 11, 12]. Overnight incubation of mesangial cells with phorbolester (TPA, 10^{-7} mol/L) completely down-regulated PKC, as shown by the inability of TPA to induce Cox-2 mRNA (Fig. 5B, gray bar compared with open bar). Pretreatment of mesangial cells with TPA also prevented the subsequent induction of Cox-2 by coincubation with platelets, indicating that PKC activation is essential for the induction of Cox-2 mRNA expression by platelet products.

Interference of dexamethasone with platelet-induced Cox-2 synthesis

Glucocorticoids interfere with Cox-2 mRNA and/or protein expression, depending on the cell type and stimulus used [30]. To investigate the effect on platelet-mesangial cell coincubation, mesangial cells were pretreated with different concentrations of dexamethasone and then stimulated with platelets (Fig. 6). Cox-2 mRNA induction detected after two hours of incubation was inhibited in a concentration-dependent manner by dexamethasone. Induced Cox-2 protein expression in mesangial cells is stable over several hours and can easily be detected after five hours. At this time point, dexamethasone was found to interfere with Cox-2 protein expression, although to a lesser extent than with mRNA synthesis.

Independent regulation of OPN and Cox-2: Up-regulation of OPN by dexamethasone

Compared with the rapid induction of Cox-2 mRNA, elevated levels of the two chemotactic proteins, MCP-1 and OPN, were observed only after four to six hours (Fig. 2). The induction of MCP-1 mRNA has been shown previously to be independent of de novo protein synthesis in mesangial cells [15]. In contrast, the preincubation of mesangial cells with an inhibitor of translation, cycloheximide, abrogated induction of OPN expression in the coculture system (Fig. 7A). The different time pattern of Cox-2 and OPN expression suggested that Cox-2 might play a role in OPN induction. To test this hypothesis, mesangial cells were incubated with PGE_2 (10 μmol/L), which resulted in the up-regulation of OPN mRNA (Fig. 7A). PGE_2 thus proved to be a potential mediator of OPN induction. The role of endogenously generated PGE_2 was assessed by inhibiting Cox activity by the nonselective Cox inhibitor ibuprofen and by interference with the induction of Cox-2 expression by the synthetic glucocorticoid dexamethasone. Both ibuprofen (10 μmol/L) and dexamethasone (10^{-6} mol/L) inhibited PGE_2 synthesis in the coculture system of platelets and mesangial cells (Fig. 7B). Dexamethasone reduced PGE_2 to basal levels, whereas ibuprofen affected basal as well as stimulated levels consistent with its inhibition of both Cox isoforms. OPN mRNA induction, however, was not inhibited by either dexamethasone or ibuprofen (Fig. 7C). Pretreatment of mesangial cells with ibuprofen did not affect the induction of OPN mRNA, whereas OPN mRNA was further up-regulated by dexamethasone. Similarly, dexamethasone enhanced up-regulation of OPN by PDGF (Fig. 8A). Even in the absence of additional stimuli, dexamethasone was able to induce a long-lasting elevation of OPN mRNA (Fig. 8 A, B). OPN is a secreted protein detectable in mesangial cell culture supernatants. Up-regulation of OPN mRNA by dexamethasone increased OPN protein levels detected after 24 hours (Fig. 8C).

DISCUSSION

A role for platelets in modulating glomerular eicosanoid metabolism during glomerular injury has been shown in several experimental in vivo studies. To further characterize the interaction between platelets and resident glomerular cells, we used an in vitro coculture system and investigated the regulation of one of the key enzymes of prostanoid synthesis, the inducible Cox isozyme (Cox-2). Regulation of this enzyme has been studied in detail in mesangial cells using individual stimuli. This approach allows detailed elucidation of signaling mechanisms but may obscure more complex regulations effective in vivo when multiple stimuli, as well as direct cell to cell interactions, act in concert to modulate gene expression.

Coincubation of freshly isolated platelets and serum-deprived mesangial cells led to a rapid interaction of the cells, resulting in the induction of gene expression in and proliferation of the mesangial cells. Compared with other coculture systems, the system platelet-mesangial cells

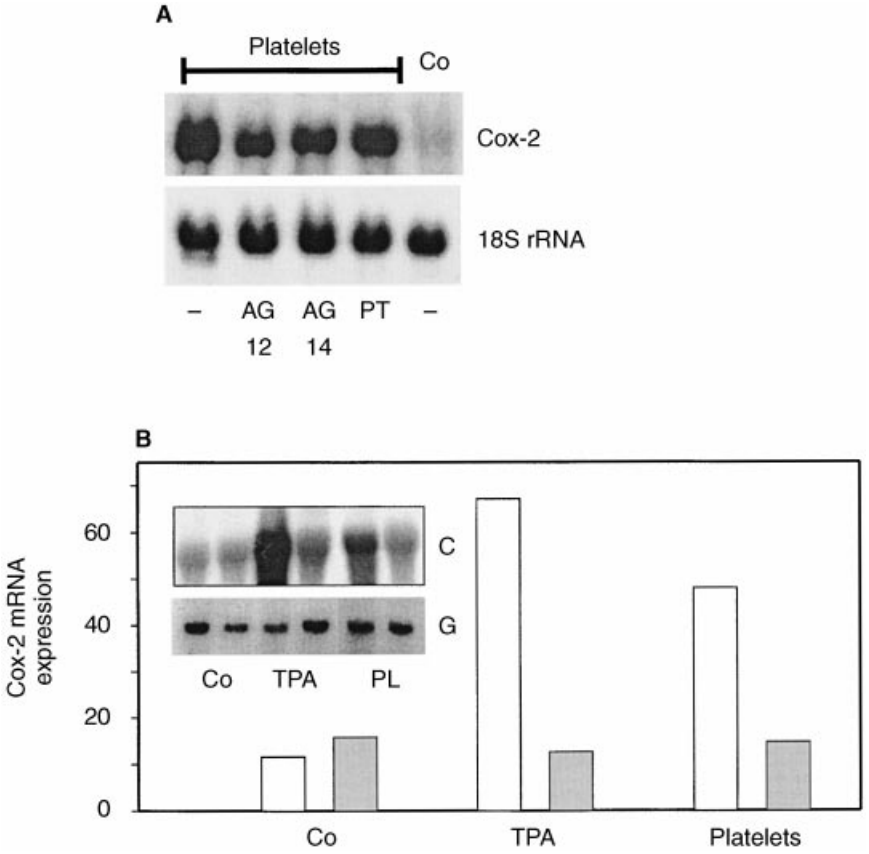


Fig. 5. Signaling pathways involved in platelet-induced Cox-2 mRNA expression. (*A*) Mesangial cells were pretreated with an inhibitor of PDGF receptor autophosphorylation AG1296 (AG12, 10 μmol/L), an inhibitor of EGF receptor autophosphorylation (AG1478, AG14, 0.1 μmol/L) for 30 minutes, or PTX (PT, 100 ng/mL) overnight and then incubated with platelets (2×10^6 cells/mL) for two hours. The blot is representative for two independent experiments. (*B*) Mesangial cells were pretreated with TPA (10^{-7} mol/L) for 20 hours to down-regulate PKC. Untreated (□) and TPA-treated (■) mesangial cells were then incubated with TPA (10^{-7} mol/L) or 5×10^6 platelets/mL (PL) for two hours. Cox-2 mRNA expression (inset, C) was corrected for GAPDH (inset, G) expression. Data shown are representative for two independent experiments.

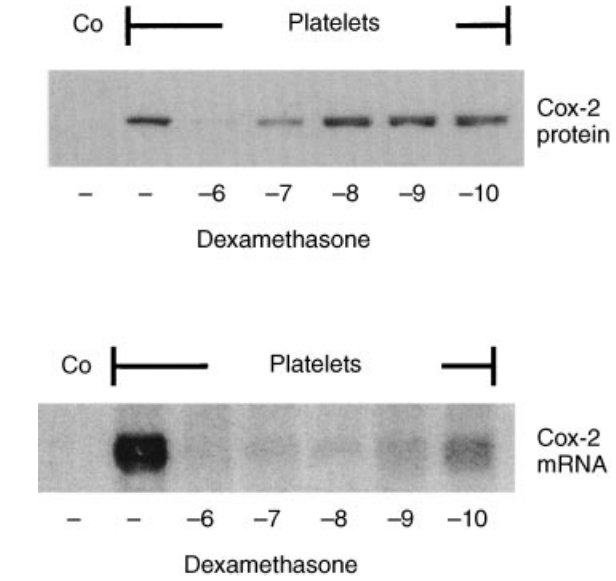


Fig. 6. Interference of dexamethasone with Cox-2 induction. Mesangial cells were pretreated with different concentrations (10^{-6} to 10^{-10} mol/L) of dexamethasone for one hour and then were incubated with 5×10^6 platelets/mL for two hours for mRNA analysis and five hours for protein analysis. Western blots were detected with an antibody directed specifically against Cox-2. Northern blots were probed with a respective cDNA. Data shown are representative for three independent experiments with similar results.

was straight forward to interpret, as only one of the cell types, the mesangial cells, is able to regulate gene expression and to proliferate.

The kinetics of Cox-2 mRNA induction were comparable to the rapid kinetics observed with stimuli such as LPA, 5-HT, PDGF, or FCS, which also induce Cox-2 mRNA within one to two hours of stimulation. This is in contrast to other types of stimuli such as interleukin-1 in mesangial cells [31] or lipopolysaccharide in macrophages [32], which take several hours to induce Cox-2 mRNA. The kinetics of Cox-2 induction are also dependent on the cell type: In monocytic cells, membrane microparticles shed from activated platelets have recently been shown to induce a long-lasting increase of Cox-2 mRNA expression [33]. With respect to time and efficiency of induction, there was very little variability between the platelets obtained from different donors.

In addition to secreting active mediators, platelets also interact with other cells by direct cell–cell contact through adhesion receptors. This type of interaction has been studied in detail, focusing on the interaction of platelets and leukocytes [34, 35], but also of platelets and endothelial cells of the blood vessel wall [36, 37], endothelial injury being a prerequisite for platelets to infiltrate sites of inflammation. To assess the importance of direct cell–cell interaction for the induction of Cox-2

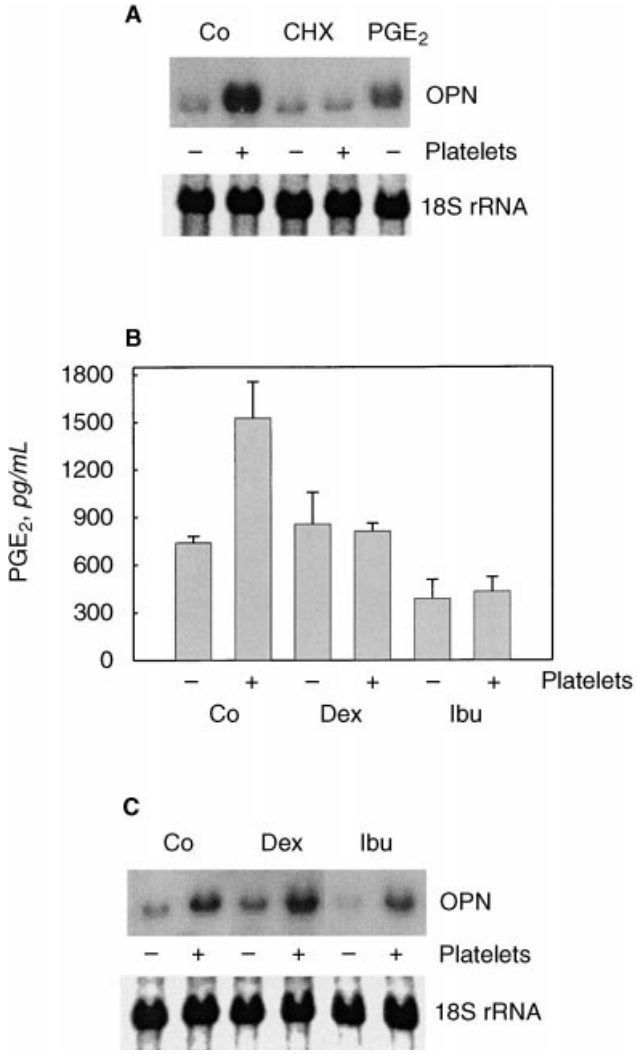


Fig. 7. Independent regulation of Cox-2 and OPN. (*A*) Mesangial cells were stimulated with platelets (2×10^6 cells/mL) for five hours with or without preincubation with cycloheximide (CHX; 10 mg/mL) for one hour. In addition, mesangial cells were stimulated with 10 mmol/L PGE_2. Northern blot analysis was performed to detect OPN mRNA. The blot is representative of three independent experiments. (*B*) Mesangial cells were preincubated with dexamethasone (Dex, 10^{-6} mol/L) or ibuprofen (10 μmol/L) for one hour and then further incubated with or without platelets for five hours. Supernatants were collected and assayed for PGE_2 by ELISA. Data are means of two experiments with duplicate determinations. (*C*) Mesangial cells were treated as described in B. OPN mRNA expression was detected by Northern blot analysis. The blot is representative of three independent experiments.

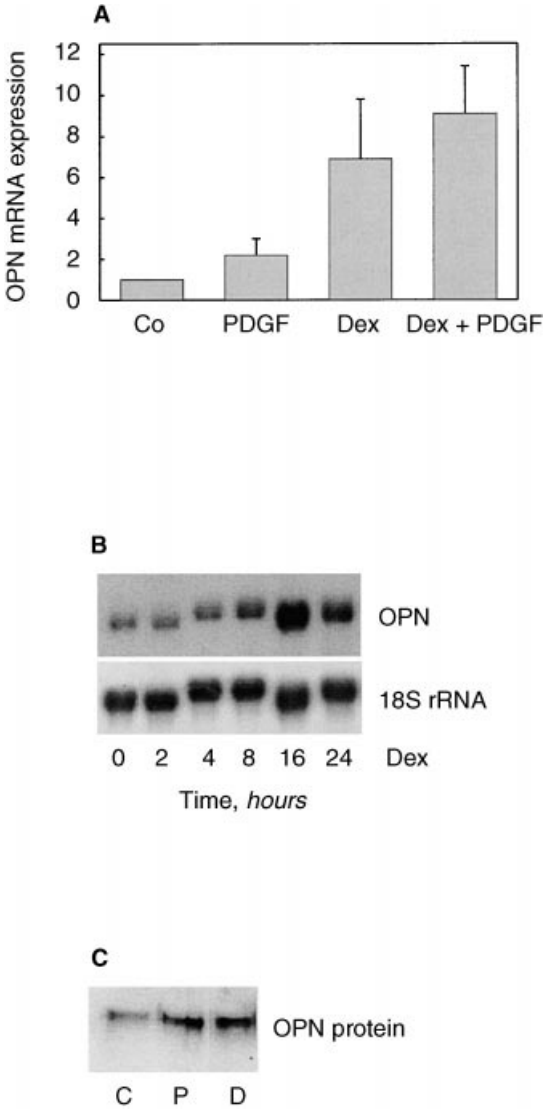


Fig. 8. Induction of OPN by dexamethasone. (*A*) Mesangial cells were incubated with PDGF (20 ng/mL) and/or dexamethasone (10^{-6} mol/L) for 24 hours. To compare the expression of OPN mRNA in four independent experiments, expression of OPN in control cells was set to 1. Data are mean ± SD. (*B*) Time course of OPN induction by dexamethasone (10^{-6} mol/L). (*C*) OPN protein expression in mesangial cell culture supernatants stimulated with PDGF (P, 20 ng/mL) or dexamethasone (D, 10^{-6} mol/L) for 24 hours.

mRNA expression, platelets were preactivated and the cell-free supernatant transferred to mesangial cells, or platelets were coincubated with mesangial cells in transwell plates, which prevented direct cell–cell interaction. In the absence of direct intercellular contact, the response to the platelet-released mediators was significantly reduced, suggesting an enhancing effect by direct cell contact possibly mediated by adhesion molecules such as P-selectin. The direct cell–cell interaction might be bidirectional, that is, stimulate the platelets to release soluble mediators or add to the activation of signaling pathways in mesangial cells.

In an attempt to characterize the contribution of individual mediators released by platelets, inhibitors were used to target specific signaling pathways. The autophosphorylation of the PDGF and the EGF receptor was inhibited by the tyrphostins AG1296 and AG1478, which have been shown recently to effectively block the induction of Cox-2 by these growth factors in mesangial cells [29]. The contribution of activators of receptors coupled

to G_i proteins, such as LPA, was assessed by pretreatment of the mesangial cells with PTX. Each of these treatments led to a partial reduction of Cox-2 expression, showing the cooperative action of different mediators in this model of cell–cell interaction. A much stronger inhibitory effect was observed when activation of PKC was prevented, which is one of the important intracellular signaling molecules involved in different signaling pathways originating from activation of receptors coupled to G proteins [11, 12], or from tyrosine kinase receptors [9]. The down-regulation of PKC by prolonged incubation with phorbol ester strongly reduced induction of Cox-2 by platelets, indicating a central role for PKC that is not overcome by the multiple signaling pathways probably activated by platelet products.

Glucocorticoids inhibit prostaglandin synthesis primarily by interfering with Cox-2 synthesis. Depending on cell type and stimulus, different molecular levels are targeted by glucocorticoids, protein synthesis, mRNA stability, or mRNA transcription of Cox-2 [30]. Pretreatment of mesangial cells with different concentrations of dexamethasone led to a concentration-dependent reduction of Cox-2 mRNA expression, with concentrations of 10^{-9} mol/L dexamethasone still being effective. The effect on Cox-2 protein levels was less pronounced, but still significant at concentrations of 10^{-7} mol/L dexamethasone. Interestingly, there was a tendency to increased Cox-2 protein levels at lower concentrations of dexamethasone. There are different possibilities to explain these apparent discrepancies between mRNA and protein levels. The stability of Cox-2 mRNA and protein largely differs in mesangial cells. Cox-2 mRNA is rapidly degraded, whereas Cox-2 protein is stable for several hours, allowing low protein levels to accumulate. Furthermore, the enhancing effects of dexamethasone on Cox-2 protein synthesis cannot be excluded, translational effects of glucocorticoids being poorly characterized. Earlier studies using single stimuli did not show a similar difference between mRNA and protein regulation by glucocorticoids, suggesting a contribution of either different types of soluble mediators present at the same time or a modulating influence of the direct cell–cell contact.

The early up-regulation of Cox-2 was followed by a delayed up-regulation of chemotactic proteins, which functionally may relate to the sequential infiltration of different types of cells of the immune system observed in glomerular immune injury with platelets and granulocytes preceding monocytes and lymphocytes [1]. The sequential induction of Cox-2 and the chemotactic proteins led to the hypothesis that up-regulation of Cox-2 and the subsequent release of prostaglandins might be involved in the up-regulation of OPN, the induction of which was dependent on de novo protein synthesis. Exogenous PGE_2 at high concentrations (10 μmol/L) was indeed able to induce OPN mRNA expression, but ibuprofen, a strong inhibitor of PGE_2 synthesis by both Cox isoforms, did not affect OPN expression. This suggested that the local concentrations of PGE_2 necessary to induce OPN expression were not achieved in the coculture system. Independent regulation of OPN and Cox-2 was shown with dexamethasone, which inhibited the expression of Cox-2, but instead of interfering, dexamethasone rather enhanced OPN expression. Induction of OPN by dexamethasone has been described in osteoprogenitor cells [38] and was related to the induction of an osteoblast-like phenotype. Regarding the proposed role of OPN in attracting monocytes to the inflamed glomerulus, down-regulation rather than up-regulation of OPN by dexamethasone had been expected. The role of OPN, however, may not be restricted to its chemoattractive properties. In myocytes and microvascular endothelial cells, increased OPN expression was related to the glucocorticoid-mediated suppression of nitric oxide synthase expression [39]. Thus, the functional consequences of glucocorticoid-mediated interference with OPN expression in the glomerulus need further evaluation.

The coculture system of platelets and mesangial cells used in the present study thus allowed us to mimic early events of glomerular inflammation. The rather small variability with platelets obtained from different donors proved this system suitable for future characterization of various aspects of platelet–mesangial cell interaction relevant in the initial phase of glomerular injury.

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